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REMARKS

THE CLAIM AMENDMENTS:

Independent claims 1, 27, and 28 have been amended to remove the word "optionally" before step (a)(iii) and to add that the heating of step (a)(iii) is to denature any double-stranded DNA or to remove mRNA secondary structure. Support for the new recitation of the removal of the mRNA secondary structure by way of heating the biological material is found in the specification at page 12, lines 6-8. Claim 1 has also been amended to remove the redundant (d) from steps (d)(iv) and (d)(v). Claim 10 has been amended to correct the dependency of that claim and claim 13 has been canceled as redundant over the recitation of step (c) of claim 1. No new matter has been added to the application with the claim amendments set forth herein.

THE LEGAL STANDARD FOR THE *PRIMA FACIE* CASE:

The *prima facie* case is a procedural tool which, as used in patent examination, means not only that the evidence of the prior art would reasonably allow the conclusion the Examiner seeks, but also that the prior art compels such a conclusion if the applicant produces no evidence or argument to rebut it. *In re Spada,* 911 F.2d 705 (Fed. Cir. 1990). If examination at the initial stage does not produce a *prima facie* case of unpatentability, then without more, the applicant is entitled to a grant of the patent. *In re Oetiker,* 977 F.2d 1443 (Fed. Cir. 1992).

The Examiner's *prima facie* case in the Office Action under reply is for obviousness. The Examiner has set forth seven separate grounds of rejection for the claims of the instant application. Each of these grounds of rejection is separately addressed and overcome in the discussion that follows.

I. CLAIM REJECTION – 35 U.S.C. § 103(a)
ANTAO ET AL. IN VIEW OF XU ET AL. ET AL.

Claims 1, 3, 4, 6-23, and 27-33 stand rejected under 35 U.S.C. § 103(a) as obvious over Antao et al. in view of Xu et al. This rejection is respectfully traversed.

To establish a *prima facie* case of obviousness, three criteria must be met: first, the prior art reference must teach or suggest the claimed combination; second, the Office must show that the ordinary artisan would be motivated to modify the reference or to combine the reference teachings; and third, there must be a showing that the ordinary artisan would have a reasonable expectation of success at arriving at the claimed combination based *solely* on the teachings of the cited prior art reference. *In re Rouffet,* 149 F.3d 1350, 1357 (Fed. Cir. 1998); *In re Vaeck,* 947 F.2d 488 (Fed. Cir.

1991). An obviousness analysis that relies upon the applicant's disclosure rather than the prior art reference is improper as being based upon an impermissible hindsight reconstruction. *In re Deuel,* 51 F.3d 1551, 1558 (Fed. Cir. 1995). In addition to the foregoing, a reference *must* be considered in whole, and portions arguing against or teaching away from the claimed invention *must* be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.,* 796 F.2d 443, 230 USPQ 416 (Fed. Cir. 1986).

As recited in claim 1, the claimed invention improves upon the bDNA and *in situ* hybridization procedures known in the prior art (*see*, specification, p.3, 2nd full ¶) by providing a highly sensitive method for *in situ* detection of a nucleic acid analyte within a sample of biological material based on bDNA hybridization comprising the steps of:

(a) preparing the sample of biological material by:

(i) immobilizing the biological material on a substrate,b

(ii) permeabilizing the substrate-bound biological material by contacting the substrate-bound biological material to a solution containing Proteinase K at a concentration of about 0.5 μg/ml to about 50 μg/ml, and

(iii) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure;

(b) contacting the biological material with a target oligonucleotide probe under hybridizing conditions, wherein at least a portion of the target probe is complementary to at least a portion of the nucleic acid analyte, so that an analyte-target probe complex is formed when the nucleic acid analyte is present in the sample;

(c) washing the biological material with a washing fluid comprising a detergent, at a temperature in the range of approximately 21 to 60°C; and

(d) detecting any analyte-target probe complex on the substrate by:

(i) contacting the washed substrate and analyte-target probe complex with a preamplifier oligonucleotide probe under hybridizing conditions, wherein a first portion of the preamplifier probe is complementary to a portion of the target probe other than the portion of the target probe that is complementary to the nucleic acid analyte, thereby forming an analyte-target probe-preamplifier probe complex when the nucleic acid analyte is present in the sample,

(ii) contacting the product of step (d)(i) with an amplifier oligonucleotide probe under hybridizing conditions, wherein a first portion of the amplifier probe is

complementary to a second portion of the preamplifier probe, thereby forming an analyte-target probe-preamplifier probe-amplifier probe complex when the nucleic acid analyte is present in the sample,

(iii) contacting the product of step (d)(ii) with a label oligonucleotide probe under hybridizing conditions, wherein a portion of the label probe binds to a second portion of the amplifier probe, thereby forming an analyte-target probe-preamplifier probe-amplifier probe-label probe complex when the nucleic acid analyte is present in the sample,

(iv) labeling the analyte-target probe-preamplifier probe-amplifier probe-label probe complex with a detectable label, and

(v) detecting the presence of the label on the substrate.

With respect to the labels that may be used in the claimed method, the specification at page 14, line 17, to page 15, line 8, sets forth various probes contemplated under the invention; preferred is an AP label detected with an AP substrate using bright field or fluorescence microscopy (page 14, line 30; page 15, lines 6-8; and claims 16 and 17).

Antoa et al. teaches the use of an *in situ* bDNA assay for RNA and DNA targets in individual cells or tissue sections (p.83). As explained in the prior Amendment (filed on December 30, 2003) and as admitted by the Examiner, Antao et al. does not teach or suggest the following: (i) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure; or (ii) washing the biological material with a detergent at a temperature range of 21°C to 60°C. Accordingly, it follows that Antao et al. alone does not render the claimed invention obvious.

In addition to the foregoing, Antao et al. addresses the issue of reducing background noise in the *in situ* bDNA assay disclosed therein through the incorporation of the natural nucleotides, iso-C and iso-G, which reduce background noise by reducing non-specific hybridization (p.82). Further, with respect to the use of the labeled probes that are used in the disclosed *in situ* bDNA assay, Antao et al. uses an AP-conjugated probe that is described in Collins et al., *A branched DNA Signal Amplification Assay for Quantification of Nucleic Acid Targets Below 100 Molecules/mL*, NUCLEIC ACIDS RESEARCH 25(15):2979-2984 (1997) and Urdea et al., *A Comparison of Non-Radioisotopic Hybridization Assay Methods Using Fluorescent, Chemiluminescent, and Enzyme-Labeled Synthetic Oligodeoxyribonucleotide Probes*, NUCLEIC ACIDS RESEARCH 16(11):4937-4956 (1988) (*see*, p.83 of Antao et al., i.e, the 1st sentence of the 3rd para. under the heading "Materials and Methods" and

reference 3 of Antao et al.).[1] Antao et al. does ***not*** teach or suggest that the labeled probe is haptenized; in fact, the haptenized probes are not mentioned in Antao et al.

Xu et al. does not correct the deficiencies of Antao et al. for the reasons that follow.

Xu et al. teaches *in situ* hybridization of mRNA with hapten-labeled probes, such as digoxigenin-UTP, fluorescein-UTP, or biotin-UTP. The Examiner asserts that Xu et al. teaches the use of a detergent to reduce background noise of the sample; however, the Examiner's interpretation of Xu's use of the detergent to reduce background noise is not so readily translated to the present invention. Referring the Examiner's attention to page 89 of Xu et al., it is noted therein, that the detergent disclosed therein is used to reduce background that is caused from ***non-specific binding of anti-hapten antibody***.

Because Antao et al. does not mention the use of hapten-labeled probes and does not mention that the hapten-labeled probes cause a background problem in the *in situ* bDNA assay disclosed therein, the ordinary artisan reading only Antao et al. would have no reason to turn to the teachings in Xu et al. regarding the reduction of noise caused by the non-specific binding of the hapten-labeled probes. Further, because the washing protocol of Xu et al. is taught strictly in relation to the reduction of non-specific binding to the hapten-labeled probes by anti-hapten antibody, the ordinary artisan would not expect that the detergent would have any successful effect on the non-specific hybridization of the *in situ* bDNA assay of Antao et al.

In addition to the foregoing, applicants also note that like Antao et al., Xu et al. does not teach or suggest heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure.

Because the hypothetical combination of Antao et al. in view of Xu et al. does not render the claimed invention obvious, applicants respectfully request reconsideration and withdrawal of this rejection.

II. CLAIM REJECTION – 35 U.S.C. § 103(a) SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., AND XU ET AL.

Claims 1, 3, 4, 6-13, 16, 17, and 20-27 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al.. This rejection is respectfully traversed.

Schaeren-Wiemers et al. teaches a non-radioactive *in situ* hybridization assay with digoxigenin ("DIG")-labeled cRNA probes for localization of selected mRNA species in tissue sections and

[1] The two cited references are attached to this response.

cultured cells from the central nervous system. As the Examiner notes, Schaeren-Wiemers et al. does ***not*** teach or suggest the following: (i) using bDNA hybridization detection means for an *in situ* hybridization assay; (ii) using proteinase K for permeabilizing the biological material; or (iii) the post-hybridization washing of the biological material with a detergent at temperatures of 21°C to 60°C. In addition to the foregoing, Schaeren-Wiemers et al. also does not teach or suggest the following: (iv) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure. Accordingly, as a primary reference, Schaeren-Wiemers et al. does not render the claimed invention obvious.

As secondary references, the Examiner cites the following references:

(1) Cao et al. for the teaching of non-isotopic *in situ* hybridization technique for detection of nucleic acid in archived sputum specimens using *in situ* bDNA technology;

(2) Nolte for the teaching of bDNA signal amplification for direct quantification of nucleic acid sequence in clinical specimens and for the suggestion that bDNA may be used for *in situ* hybridization assays;

(3) Decimo et al. et al. for the teaching of basic conditions for conducting *in situ* hybridization assays; and

(4) Xu et al. for the teaching of *in situ* hybridization of mRNA with hapten labeled probes where the labeled samples are washed at 55-65°C, 0.1 to 0.5 μg of probes are used for hybridization, and Triton, NaCl, and KCl are included in the washing steps.

The Examiner's secondary references do not correct the deficiencies of Schaeren-Wiemers et al. for the reasons that follow.

The Cao et al. abstract, which is referenced on page 3 of the specification, teaches the application of a bDNA assay to *in situ* hybridization to detect mRNA. As noted in the specification, the sensitivity of this assay is low and thus, the claimed invention was designed in part to improve upon the low sensitivity of the Cao et al. assay. Notwithstanding the foregoing, the Cao et al. abstract does not provide any procedures on the preparation of the sample for the *in situ* bDNA procedure. In particular, Cao et al. does not teach or suggest any of the following: (a) the use of proteinase K for permeabilizing the biological material; (b) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure; and (c) the post-hybridization washing of the biological material with a detergent at temperatures of 21°C to 60°C. Because Schaeren-Weimers et al. in view of Cao et al. do not teach or suggest (a)-(c), it follows that the ordinary artisan could not arrive at the claimed invention solely through a reading of Schaeren-Weimers et al. in view of Cao et al.

With respect to Nolte, first, applicants would like to note that the Examiner's "Response to Arguments" does not accurately describe applicants arguments in the Amendment of December 30, 2003; there, applicants noted that the suggestion in Nolte does not provide any guidance on how the bDNA procedure disclosed therein may be applied to ***in situ* hybridization**. The Examiner incorrectly characterizes applicants' arguments as stating that Nolte provide no guidance for bDNA hybridization; applicants did not say this. Second, with respect to the fleeting suggestion in Nolte that the bDNA assay may be used in *in situ* hybridization, applicants note, as they did in the prior Amendment, that this disclosure is minimal at best and provides no specific procedures on how the bDNA assay is applied to *in situ* hybridization. Notwithstanding the foregoing, Applicants acknowledge that Nolte et al. describes the bDNA procedure at Figure 1; however, this teaching does not compensate for the missing teachings from Schaeren-Wiemers et al. and Cao et al., which are as follows: (a) the use of proteinase K for permeabilizing the biological material; (b) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure; and (c) the post-hybridization washing of the biological material with a detergent at temperatures of 21°C to 60°C. Because Schaeren-Weimers et al. in view of Cao et al. and Nolte do not teach or suggest (a)-(c), it follows that the ordinary artisan could not arrive at the claimed invention solely through a reading of Schaeren-Weimers et al. in view of Cao et al. and Nolte

Decimo et al. teaches the conditions for conducting *in situ* hybridization, including the use of 1 μg/mL of proteinase K to "improve the penetration of the probe" (page 191, 2nd sentence under the heading 4.4) and a post-hybridization washing step conducted at 55°C for approximately 45 minutes. Decimo et al. does not teach or suggest the following: (1) the use of a detergent in the post-hybridization washing step; and (2) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure. Because Schaeren-Weimers et al. in view of Cao et al., Nolte, and Decimo et al. do not teach or suggest (1) and (2), it follows that the ordinary artisan could not arrive at the claimed invention solely through a reading of Schaeren-Weimers et al. in view of Cao et al., Nolte, and Decimo et al.

As noted above, Xu et al. teaches the use of a detergent to reduce the non-specific binding of hapten-labeled probes by anti-hapten antibody. Xu et al. does not teach or suggest heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure. Because Schaeren-Wiemers et al. in view of Cao et al., Nolte, and Xu et al. do not teach or suggest heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to

remove mRNA secondary structure, it follows that the ordinary artisan could not arrive at the claimed invention solely through a reading of Schaeren-Weimers et al. in view of Cao et al., Nolte, Decimo et al. and Xu et al. Furthermore, because Cao et al., Nolte, Decimo et al. and Xu et al. do not provide any motivation for the ordinary artisan to heat the permeabilized biological material to a temperature and for a time period sufficient effective to denature any double-stranded DNA or to remove mRNA secondary structure, the ordinary artisan would not have any expectation of arriving at the claimed invention merely by reading the disclosures of Schaeren-Weimers et al. in view of Cao et al., Nolte, Decimo et al. and Xu et al.

Because none of the cited references, alone or in combination, lead the ordinary artisan to a highly sensitive bDNA *in situ* hybridization assay as claimed in independent claims 1 and 27, it follows that the hypothetical combination of Schaerens-Weiner et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. does not render claims 1, 3, 4, 6-13, 16, 17, and 20-27 obvious; accordingly, applicants respectfully request reconsideration and withdrawal of this rejection.

III. CLAIM REJECTION – 35 U.S.C. § 103(a) SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., XU ET AL., AND SARTO ET AL.

Claim 5 stands rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al., and Sarto et al. This rejection is respectfully traversed.

Claim 5 depends from claim 1 and recites a concentration of proteinase K from about 5μg/mL to about 20 μg/mL.

Schaeren-Wieimers et al., Cao et al., Nolte, Decimo et al., and Xu et al. are discussed above. The Examiner cites Sarto et al. for the additional teaching of the use of 2.5μg/mL to 10 μg/mL of proteinase K to treat cells for an *in situ* hybridization assay.

Because Schaeren-Wieimers et al., Cao et al., Nolte, Decimo et al., and Xu et al. do not render obvious the method as claimed in independent claim 1, the additional teaching of Sarto et al. will not serve to render claim 5 obvious. Accordingly, applicants respectfully request reconsideration and withdrawal of this rejection.

IV. CLAIM REJECTION – 35 U.S.C. § 103(a) SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., AND KERN ET AL.

Claims 14 and 15 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al., and Kern et al. This rejection is respectfully traversed.

As a preliminary matter, applicants note that the Examiner does not include Xu et al. in the listing of references for this rejection; however, because Xu et al. is mentioned in the text of the rejection, applicants are taking the position that this was just an oversight by the Examiner and are including Xu et al. in this traversal.

Claim 14 depends from claim 1 and recites the use of approximately 1 fmole to about 10 pmoles of preamplifier probe.

Claim 15 depends from claim 14 and recites the use of approximately 1 fmole to about 10 pmoles of the amplifier oligonucleotide probe.

Schaeren-Wieimers et al., Cao et al., Nolte, Decimo et al., and Xu et al. are discussed above. The Examiner cites Kern for the additional teaching of 0.7 fmole/μL of preamplifier and 1 fmol/μL of amplifier in the bDNA assay disclosed therein.

Because Schaeren-Wieimers et al., Cao et al., Nolte, Decimo et al., and Xu et al. do not render obvious the method as claimed in independent claim 1, the additional teaching of Kern et al. will not serve to render claims 14 and 15 obvious. Accordingly, applicants respectfully request reconsideration and withdrawal of this rejection.

V. CLAIM REJECTION – 35 U.S.C. § 103(a) SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL. OR XU ET AL., AND SIADAT-PAJOUH

Claims 18 and 19 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al., and Siadat-Pajouh et al. This rejection is respectfully traversed.

As a preliminary matter, applicants note that the Examiner uses the language "Decimo...or...Xu" in this rejection; however, because the Examiner implies the use of "Decimo...and...Xu" in the text of the rejection, applciatns are taking the position that the Examiner was intending this rejection to include both Decimo et al. and Xu et al. rather than one but not the other.

Claim 18 depends from claim 1 and recites that the method of claim 1 has a sensitivity sufficient to detect from 1 to about 10 copies of the nucleic acid analyte.

Claim 19 depends from claim 18 and recites that the method of clam 1 has a sensitivity sufficient to detect from 1 to about 2 copies of the nucleic acid analyte.

Schaeren-Wiemers et al., Cao et al., Nolte, Decimo et al., and Xu et al. are discussed above. The Examiner cites Siadat-Pajouh et al. for the teaching of a method of detecting single copy HPV genes with *in situ* hybridization.

Because Schaeren-Wieimers et al., Cao et al., Nolte, Decimo et al., and Xu et al. do not render obvious the method as claimed in independent claim 1, the additional teaching of Siadat-Pajouh et al. will not serve to render claims 18 and 19 obvious. Accordingly, applicants respectfully request reconsideration and withdrawal of this rejection.

VI. CLAIM REJECTION – 35 U.S.C. § 103(a)
SIADAT-PAJOUH IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., AND XU ET AL.

Claims 28-35 stand rejected under 35 U.S.C. § 103(a) as obvious over Siadat-Pajouh et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. This rejection is respectfully traversed.

Siadat-Pajouh et al. relates to a fluorescence *in situ* hybridization technique to detect one to five copies of the human papillomavirus ("HPV") genome using DIG-labeled oligonucleotides. The Examiner acknowledges that Siadat-Pajouh does ***not*** teach using bDNA for signal amplification and detection of nucleic acid analytes *in situ* (Office Action, p.16). In addition, Siadat-Pajouh does not teach or suggest (i) using proteinase K for permeabilizing the biological material; (ii) heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure; or (iii) post-hybridization washing of the biological material with a detergent at temperatures of 21°C to 60°C (*see*, "Materials and Methods" starting on p.1504, col. 1 to p.1505, col. 2).

The teachings of Cao et al., Nolte, Decimo et al., and Xu et al. are discussed above. Because none of the cited references alone or in combination teach or suggest heating the permeabilized biological material to a temperature and for a time period effective to denature any double-stranded DNA or to remove mRNA secondary structure, it follows that the hypothetical combination of Siadat-Pajouh et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. does not render claims 28-35 obvious.

CONCLUSION:

The foregoing analysis demonstrates that the Office has not established a *prima facie* case of obviousness against the claimed invention. Because the Office's *prima facie* case has been overcome with this response, it follows that applicants are entitled to a patent grant for the invention described in the instant application. *See, In re Oetiker, supra.*

Should the Examiner have any questions concerning this response, she is welcome to telephone the undersigned attorney at 650-330-4913 or at canaan@reedpatent.com.

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A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml

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ABSTRACT

The branched DNA hybridization assay has been improved by the inclusion of the novel nucleotides, isoC and isoG, in the amplification sequences to prevent non-specific hybridization. The novel isoC, isoG-containing amplification sequences have no detectable interaction with any natural DNA sequence. The control of non-specific hybridization in turn permits increased signal amplification. Addition of a 14 site preamplifier was found to increase the signal/noise ratio 8-fold. A set of 74 oligonucleotide probes was designed to the consensus HIV POL sequence. The detection limit of this new HIV branched DNA amplifier assay was ~50 molecules/ml. The assay was used to measure viral load in 87 plasma samples of HIV- infected patients on triple drug therapy whose RNA titers were <500 molecules/ml. In all 11 patients viral load eventually declined to below the detection limit with the new assay.

INTRODUCTION

Quantitative hybridization assays based on branched DNA signal amplification are widely used to monitor patients on antiviral therapy for human immunodeficiency virus (HIV), hepatitis C (HCV) and hepatitis B (HBV) and to stratify patients for therapy (1–7). They have also been used to predict time to onset of AIDS (8–10) and to establish the kinetics of HIV production and destruction, which has led to new insights into the mechanisms of pathogenesis (11,12). The most important characteristics of these hybridization assays are sensitivity, wide dynamic range, and precise and accurate quantification. The branched DNA hybridization assay pictured in Figure 1 employs linear signal amplification rather than exponential amplification of target.

A family of oligonucleotides called capture extenders (CEs) is used to capture the target to the solid support. The target is labeled by virtue of binding a large number (typically >30) of target-specific oligonucleotides called label extenders (LEs). In the first generation assay the LE probes bind a branched DNA amplifier (bDNA), which in turn binds many alkaline phosphatase probes. In the second and third generation assays, the LE probes bind pre-amplifiers, which in turn bind many amplifiers. The result is stronger signal amplification and lower detection limits. In all versions of the assay, the linearly amplified signal is directly related to the number of targets present in the original sample. This first generation bDNA assay has been shown to quantify nucleic acid targets accurately and precisely between ~10 000 and 10 000 000 molecules; assays for HIV, HCV and HBV have been developed (13–17). The second generation HIV bDNA assay has a quantitative detection limit of 500 molecules (18).

The recent introduction of HIV protease inhibitors has driven viral loads to below even the detection limits of the second generation HIV bDNA assay (1,2). The first and second generations of the assay were limited by non-specific hybridization (NSH) between the amplification sequences and other nucleic acids. Short regions of hybridization between any member of the amplification system (alkaline phosphatase probe, amplifier or preamplifier) and any non-target nucleic acid sequence will lead to amplification of background. Capture probes (CPs), CEs and sample nucleic acids are all sources of this background hybridization. The purpose of this study was to examine the effect of redesigning the amplification molecules by incorporation of the non-natural bases, isocytidine and isoguanosine, to reduce their hybridization potential to all non-target nucleic acids. If target-specific signal amplification is accomplished without a concomitant amplification of the background from non-target molecules, then sensitivity should be greatly improved.

MATERIALS AND METHODS

Clinical specimens

Blood was collected in EDTA tubes. Plasma was prepared and stored at –80°C until use and virus was concentrated by centrifugation as described (18).

Oligonucleotide probe design

A consensus HIV POL sequence was generated from the GenBank database using sequences from six subtypes, A–F, and sectioned

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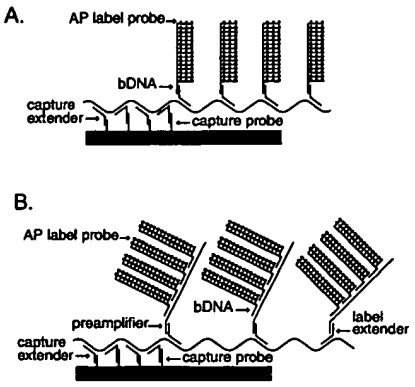

Figure 1. Basic bDNA assay components. (A) First generation assay; (B) second and third generation assays. The preamplifier (heavy lines) is unique to the second and third generation assays.

into 26 nucleotide (nt) fragments, resulting in an average dissociation temperature (T_d) of 72°C. This created 110 possible oligonucleotide probes. A computer program (S.Bushnell *et al.*, in preparation) was used to calculate degeneracy, equilibrium melting temperature (T_m) and dissociation temperature for each oligonucleotide. An initial probe set was selected based on the following criteria: $T_m > 63$°C, $T_d > 63$°C, and degeneracy less than four sites. The program then calculates the homology between the initial probe set and the amplification sequences. It selects probes with the lowest homology (below a user-defined cutoff) to the amplification sequences to be CEs. Finally, probes with the lowest homology to the CP and the CEs are then selected to be LEs. The resulting probe set consisted of 54 LE and 20 CE probes.

Universal sequence design

Sequences for the preamplifier, amplifier and alkaline phosphatase probe were redesigned to contain ~30% isobases [5-methyl-2′-deoxyisocytidine (isoC) and 5-methyl-2′-deoxyisoguanosine (isoG)]. The sequences were designed to have no secondary structure longer than a trimer, no interactions with any sequence longer than a trimer except with their complements, and to have a melting temperature in excess of 80°C at 1 nM in 3× SSC. The sequences chosen are listed in Table 1.

Oligonucleotide target design

To measure the improvement in sensitivity with each layer of amplification in the bDNA assay, oligonucleotide targets were designed to hybridize to the CP on the microtiter well and serve as targets for the amplification system. The sequences are listed in Table 2.

Preamplifier synthesis

Eight oligonucleotides were enzymatically ligated essentially as described (18) to produce a molecule with 14 repeat sequences and one leader sequence.

Amplifier synthesis

A 15×2 branched DNA was constructed essentially as described (19). An arm consisting of two copies of the amplifier repeat was ligated onto a comb containing one copy of the amplifier leader sequence.

Alkaline phosphatase probe

An alkaline phosphatase probe was prepared essentially as described (20).

Table 1. Sequences used in the system 8 bDNA amplification assay, which is pictured in Figure 1

Function (copies)	Sequence	Complement
LE tail (1)	d(JTATJCGCJCTGFTATJCCG)	Preamplifier leader (1)
Preamplifier repeat (14)	d(TCFACGJCFCTAJGGAFAAFG)	Amplifier leader (1)
Amplifier repeat (30)	d(AGTFAJCGCFGTAFCAAJTJC)	Alkaline phosphatase probe (1)

The sequences labeled 'leader' appear once in the indicated construct, while sequences labeled 'repeat' appear the indicated number of times. The tail on the LE is a single sequence. F, isoC; J, isoG.

Table 2. Oligonucleotide target sequences

Oligonucleotide	Sequence	Complement
1	d(AGTFAJCGCFGTAFCAAJTJCQCTCTTGGAAAGAAAGTGAAGTGT)	Alkaline phosphatase probe
2	d(TCFACGJCFCTAJGGAFAAFGQCTCTTGGAAAGAAAGTGAAGTGT)	Amplifier
3	d(JTATJCGCJCTGFTATJCCGQCTCTTGGAAAGAAAGTGAAGTGT)	Preamplifier

All three oligonucleotides possess the same 23 nt 3′ terminal sequence that allows them to hybridize to the CP on the microtiter wells. Oligonucleotide 1 is detected by direct hybridization with the alkaline phosphatase probe; oligonucleotide 2 is detected by hybridization with the bDNA amplifier, which hybridizes to the alkaline phosphatase probe; oligonucleotide 3 is detected by hybridization with the preamplifier, which hybridizes to the amplifier, which hybridizes to the alkaline phosphatase probe. F, isoC; J, isoG; Q, triethyleneglycol spacer.

Capture wells

Polystyrene capture wells were prepared essentially as described (21).

bDNA diluents

Capture diluent. 127 mM LiCl, 5% lithium lauroyl sulfate, 9 mM EDTA, 50 mM HEPES (pH 7.5), 0.05% hespan (DuPont Pharmaceuticals), 0.05% ProClin 300 (Supelco), 0.2% casein (Research Organics, Hammarsten quality).

Amplifier diluent. Prepared by incubating 50% horse serum with 0.5 mg/ml proteinase K in 5× SSC, 1.3% SDS, 6 mM Tris–HCl at 65°C for 2 h and then adding 6 mM phenylmethylsulfonyl fluoride, 0.05% sodium azide, 0.05% ProClin 300 and 10% dextran sulfate (500 000 MW; Pharmacia).

Alkaline phosphatase diluent. Prepared by adding 17 mM $MgCl_2$, 0.85 mM $ZnCl_2$, and 0.85% Brij 35 to amplifier diluent without dextran sulfate.

System 8 bDNA assay procedure

System 8 is a detection system employing isoC and isoG in the preamplifier, amplifier and alkaline phosphatase probe. Viral pellets were resuspended in capture diluent and solubilized at 63°C for 2 h prior to overnight incubation at 45°C in capture wells. Three serial incubations were done at 45°C with no cool down between steps: preamplifier (5 fmol/50 µl in amplifier diluent) for 30 min, amplifier (5 fmol/50 µl in amplifier diluent) for 30 min, and ap probe (10 fmol/50 µl in alkaline phosphatase diluent) for 15 min. After each incubation step, the wells were washed twice with wash A (18). An additional three washes with wash D (18) were done after the alkaline phosphatase step, prior to incubation with substrate (18) in the Chiron luminometer. After 30 min at 37°C the luminescence was measured in relative light units (RLU).

Calculation of detection limit

Let D = detection limit, T = Target level, S_T = signal at T, and N = assay noise. Detection limit is defined as the target level at which $\Delta = 0$. The Δ function is defined by:

$$\Delta = (S - 2)(CV_s \times S) - (N - 2)(CV_n \times N)$$

where CV is the coefficient of variation, $CV_s \times S = \sigma_s$ = standard deviation of S, and $CV_n \times N = \sigma_n$ = standard deviation of N. Let S_d = signal at the detection limit, then

$$S_d = [N(1 + 2Cv_n)]/(1 - 2CV_s)$$

At the detection limit, $CV_s \sim CV_n \sim CV_{assay}$, the average CV of the entire assay. This is true because assays to measure detection limit use points clustered about the detection limit (i.e., all target levels are close to the detection limit). Thus $S_d \sim [N(1 + 2CV_{assay})]/(1 - 2CV_{assay})$. At any point along the S – N curve versus T, one can estimate D as approximately equal to $T(S_d - N)/(S_T - N)$. The detection limit is estimated for each target level. An average and standard deviation of the detection limit estimated at three or more target levels are then calculated. Linearity, L, was estimated at two or more target levels, T_1 and T_2, as

$$L = 100\% \times [(S_1 - N)/(S_2 - N)]/(T_1/T_2).$$

A perfectly linear dose-response curve = 100% linearity.

RESULTS

Control of non-specific hybridization by redesign of the amplification sequences with isoC and isoG

To improve the sensitivity of the bDNA assay, while maintaining the precision and accuracy of quantification and the wide dynamic range, all of the amplification sequences were redesigned. In the new design, termed 'system 8 bDNA', approximately every fourth nucleotide of the preamplifier, amplifier and alkaline phosphatase probe is either isoC or isoG (Table 1), which attenuates the hybridization of these molecules to natural sequences (22,23). IsoC and isoG base pair with each other, but not with any of the four natural bases (24). Sequences containing isoC–isoG base pairs are ~2°C more stable per base pair than their C–G congeners (24).

To test the ability of amplification sequences containing isoC and isoG to control NSH, 100 fmol of 12 CEs [to hepatitis G virus (HGV) RNA] were bound individually to microwells and incubated with natural sequence amplifier and alkaline phosphatase probe or system 8 (isoC and isoG) amplifier and alkaline phosphatase probe. Binding was measured by dioxetane chemiluminescence (25,26) RLU. The results are shown in Table 3.

The RLU of the no CE control, which represents assay NSB (non-specific binding), was subtracted from the RLU of individual CE probes to highlight sequence-specific binding. The NSB of the natural sequence amplifier and alkaline phosphatase probe was 10.9 RLU and the NSB of the isoC, isoG amplifier and alkaline phosphatase probe was 3.0 RLU. NSH Background < 5 RLU is not significant in view of the natural sequence NSB value. The natural sequence amplifier showed sequence-specific background with seven of the 12 oligonucleotide probes, ranging from 1200 to 12 RLU. The 1200 RLU background of probe HGV.225 is the highest ever observed. Computer modeling of the background with this probe suggests that the amplifier repeat sequence forms two 5mer hybrids with the CE separated by a 5 nt bulge. The background is most likely caused by the binding of multiple amplifier repeat sequences to multiple CE probes on the well surface (polyvalent binding).

Table 3. Control of NSH with amplifier sequences containing isoC and isoG

Capture extender	Natural sequence: NSH (RLU)	IsoC,isoG: NSH (RLU)
HGV.225	1227	0
HGV.247	117	0
HGV.181	90	0
HGV.115	36	0
HGV.159	33	0
HGV.27	14	1
HGV.71	12	0
HGV.93	5	0
HGV.5	3	0
HGV.137	2	0
HGV.203	2	0
HGV.269	–2	0
Control (no CE probe)	0	0

The indicated CE probe (100 fmol) was bound to microwells and incubated with either natural sequence amplifier and alkaline phosphatase probe or the isoC,isoG amplifier and isoC,isoG alkaline phosphatase probe. Hybrids were quantified by dioxetane chemiluminescence in RLU. The value of the no CE probe control, which represents NSB was subtracted from the total noise to yield the NSH of each CE probe.

Table 4. Improvement of the signal/noise ratio by employing multiple layers of amplification in the system 8 bDNA assay

Amplification molecules	Signal (RLU)	Noise (RLU)	Signal/Noise
Alkaline phosphatase probe only	2.6	0.4	5.5
Amplifier and alkaline phosphatase probe	10.3	0.5	19.6
Preamplifier, amplifier and alkaline phosphatase probe	93.2	0.6	154.3

Oligonucleotide targets (5 attomol), listed in Table 2, complementary to either the alkaline phosphatase probe, the amplifier or the preamplifier were hybridized to the CP on microtiter wells. The oligonucleotide targets were detected with the indicated amplification molecules and the hybridization signal measured by dioxetane chemiluminescence in RLU.
Noise = RLU associated with no oligonucleotide target.

The system 8 amplifier and alkaline phosphatase probe showed no background (<2 RLU) with any of the 12 CE probes. The absence of NSH to system 8 amplifier and alkaline phosphatase probe was also observed for 30 CE probes designed to five different cytokine mRNAs; the natural amplifier again showed sequence-specific variation of background, ranging from 35 to 0 RLU (data not shown).

Sensitivity improves with each additional layer of amplification

Control over NSH with isoC and isoG (system 8 amplification) should in principle allow the use of larger preamplifiers and larger amplifiers or the use of multiple layers of amplification to improve sensitivity since signal can be augmented without equal amplification of noise. To test this concept, 5 attomol of three different isoC, isoG oligonucleotide targets (Table 2) were hybridized to the CP on microtiter wells. Target number 1 was detected by hybridization with the alkaline phosphatase probe; target number 2 was detected by hybridization with the amplifier, followed by the alkaline phosphatase probe; target number 3 was detected by hybridization with the preamplifier, followed by the amplifier, followed by the alkaline phosphatase probe. The sensitivity was determined by dioxetane chemiluminescence. The results are shown in Table 4.

Detection of 5 attomol of oligonucleotide target with alkaline phosphatase probe had an S/N of 5.5; a two-layered amplification had an S/N of 19.6; a three-layered amplification had an S/N of 154.3. By extension, it is likely that a four layered system will increase the sensitivity of the three-layered system. A 30 site bDNA amplifier improved sensitivity another 1.4-fold (data not shown) as compared to the 15 site bDNA amplifier used in this experiment.

Detection of 60 molecules of HIV RNA with system 8 bDNA assay

To test the performance of system 8 detection of an RNA target, the HIV POL RNA sequence was chosen.

An HIV RNA standard curve was prepared by serial dilution of virus in negative human plasma and was titered against an HIV POL RNA standard quantified by phosphate analysis and confirmed by OD_{260} and hyperchromicity (27). The samples were hybridized overnight in quadruplicate to the oligonucleotide probes in microtiter wells. The captured RNA was detected by dioxetane chemiluminescence following system 8 amplification. The results are shown in Table 5.

Table 5. System 8 bDNA HIV RNA molecular detection limit

Experiment no.	HIV RNA (molecules)	Signal (RLU)	Linearity (%)	Detection limit (molecules)
1	1440	7.2	97	91
	720	4.5	91	88
	360	3.2	100	80
	180	2.4		80
	0	1.6		85 ± 6 (average)
2	3200	20.3	101	45
	320	3.1	119	45
	160	2.0		53
	0	1.2		48 ± 5 (average)
3	400	5.5	82	55
	200	4.2	66	45
	50	2.9		29
	0	2.1		43 ± 13 (average)

In three independent experiments, serial dilutions of HIV (prepared from frozen plasma and quantified against the standard RNA transcript) were incubated overnight in microwells with 20 CE and 54 LE oligonucleotides and the signal was amplified with the system 8 preamplifier, amplifier and alkaline phosphatase probes. Dioxetane chemiluminescence was measured in RLU.
Noise = no HIV (negative human plasma). Linearity (dose–response) and Detection limit are defined in Materials and Methods. Signals are the average of four determinations.

The three assays each have ~5% CV. The linearity and detection limit were calculated as described in Materials and Methods for each target level T.

The average detection limit of the three experiments is 59 ± 23 molecules: the percent CV of the detection limits reflect the dose-response. The better the linearity of S – N versus T, the lower the percent CV of the detection limit. A perfectly linear dose–response = 100% linearity. The curves thus show precise quantification down to the limit of 60 molecules/ml.

Utility of system 8 bDNA assay in monitoring HIV-infected patients on triple drug therapy

The system 8 bDNA assay was used to measure viral load in 87 samples from HIV-infected patients on various therapy regimens, including triple drug therapy consisting of Nelfinavir, AZT and 3TC. Figure 2 summarizes the results of this study.

The viral load of the 11 patients showed a nearly monotonic decline to below the assay detection limit during the treatment. A

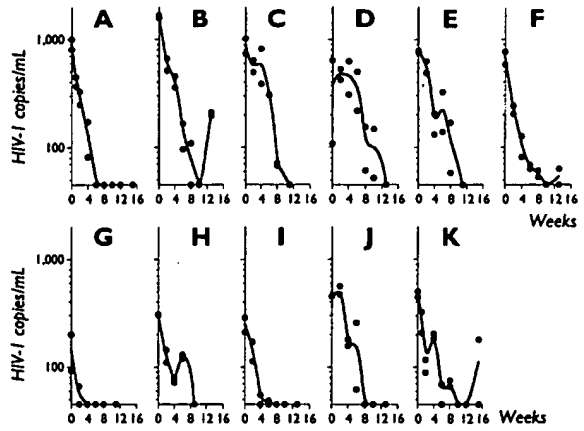

Figure 2. Quantification of HIV RNA/ml in 11 patients over time. Plasma was collected from the patients at the indicated time intervals and stored at –80°C in David Ho's laboratory. Duplicate 1 ml samples were concentrated by centrifugation and system 8 bDNA assays were run at Chiron. Serial dilutions of virus (quantified against standard HIV POL RNA) were used as a standard curve, which was run in quadruplicate. Each point shown on the graph is a single determination.

total of 47 samples (54%) quantified above the detection limit and 78 samples (90%) quantified above zero. Only the development of a still more sensitive assay will be able to validate the positivity of the 31 samples quantifying between 0 and the detection limit. In spite of the fact that the average sample contained ~130 virions/ml, the average CV (of the RLU) of the duplicate samples was only 14%.

The HIV 2.0 bDNA assay was previously performed on these samples, all of which were below the detection limit of 500 molecules/ml. This is in good agreement with the current study in which 76 out of 87 samples quantified <500 molecules/ml and 10 out of 11 quantified between 500 and 1000 molecules/ml.

DISCUSSION

A more sensitive branched DNA assay has been developed to quantify nucleic acid targets <100 molecules/ml. This system 8 bDNA assay employs isoC and isoG nucleotides in the amplification molecules to reduce backgrounds and allow for stronger amplification. System 8 amplification was used to show that triple therapy is at least eight times more effective (<60 molecules/ml) than determined using the previous version of the branched DNA assay (<500 molecules/ml). If 10 ml samples were concentrated before the assay, the assay could theoretically detect ~6 HIV RNA/ml or 3 virions/ml.

In theory the system 8 bDNA assay can be made considerably more sensitive not only by increasing volume, but also by increasing the S/N ratio. Most of the background is coming from LE NSB and amplifier NSB (data not shown). By using cruciform LEs, a design in which two LE probes must bind the target in the correct orientation to bridge the preamplifier, most of the LE NSB can in theory be removed (18). By finding more effective blockers for the solid phase or by redesigning the branched DNA molecule or the solid phase itself, much of the amplifier's NSB can be removed. Alternatively, by using sequences of reduced complexity (such as trinucleotide repeats), lower concentrations of the amplification molecules can be used during hybridization, resulting in reduced NSB. Additional layers of amplification can also be added.

A prototype system 8 bDNA assay has also been developed to quantify HCV RNA in plasma. A total of 12 2′-*O*-methyl probes (eight LE and four CE) were designed to the well-conserved 5′ untranslated region of the genome. The assay has a detection limit of 200 molecules (unpublished data), which is ~50-fold better than the HCV 2.0 Quantiplex™ assay (15).

The system 8 bDNA assay should also be useful in other hybridization assays. In both filter and *in situ* hybridization assays, for example, billions of overlapping, unique oligonucleotide sequences are available for possible NSH to probes. The ability to amplify the signal without amplifying noise from hybridization of amplification molecules to sample nucleic acid sequences should greatly improve the sensitivity of these assays. Currently, *in situ* PCR is the standard for detection of single copy DNA sequences in cells (28,29); *in situ* RT–PCR has occasionally been problematic for mRNA detection (30–33). RNA targets that are partially degraded or intramolecularly crosslinked at selected sites should pose no special problems for *in situ* bDNA assays since priming and reverse transcription are not required. As in assays that target DNA, multiple oligonucleotides will be used to label target RNA in cells; failure to bind one or more of these oligonucleotides is of no real consequence. Quantification may also be possible with the *in situ* bDNA assay with proper selection of internal standards. The sensitivity of the system 8 bDNA filter and *in situ* hybridization assays should be limited mostly by the specificity of the oligonucleotide probes. Empirical selection of the best LE oligonucleotide probes and the use of the cruciform design (18) should prove most useful in optimizing specificity.

The isoC–isoG base pair may find utility in several other areas. Amplification of weak signals from immunoassays, particularly with low titer antigens on the surface of cells or within cells, should be possible. In multiplex bDNA hybridization analysis, many targets will be analyzed simultaneously using many CPs and CE sequences. The ability to control NSH of the labeling system with any of the multiple capturing systems will be critical to the success. Combinatorial fluorescence (34) can be used in multiplex applications to increase the specificity and the reliability of target detection at very low levels. With a six base code containing isoC and isoG nucleotides, it is relatively easy to construct four complete labeling systems (preamplifier, amplifier, labeled probe) that will have no cross-hybridization greater than a 3mer with each other.

In nanotechnology, DNA is a more useful framework than other biopolymers because of the regularity and the predictability of the structures that form when unique sequences are mixed together under appropriate hybridization conditions (35,36). The use of isoC–isoG in these constructions should allow more intricate structures to be formed with greater control over the specificity of the hybridizations.

Provided that isoC and isoG are not toxic, they may find utility in oligonucleotide therapeutics. The isoC–isoG base pair could be used to replace selected C–G base pairs to increase the specificity of nucleic acid aptamers (37–39) and decoys (40). This should reduce undesirable NSH of the aptamers or decoys to cellular nucleic acid sequences. The use of isoC and isoG in constructing nanomachines for delivery of therapeutics to target cells can also be envisioned.

Other base pairs that may be useful in these applications have been defined, including the κ-π base pair with three hydrogen bonds (22,23) and a stability similar to the A–T base pair (41,42). With an eight base code, sequences of even greater uniqueness and specificity can be devised for all of the above applications.

Eventually, quantification with all DNA probe assays (including quantitative PCR) will be limited by Poisson sampling error. To analyze this, the assay can be conveniently divided into three steps, each of which has an associated probability distribution and variance (σ^2): (i) pipetting the sample containing n target molecules (Poisson distribution, $\sigma P^2 = n$); (ii) capture of the targets (binomial distribution, $\sigma b^2 = nPq$), where P = probability of capture and $q = 1 - P$; and (iii) amplification and detection [a normal distribution, $\sigma ad^2 = (CV_{ad} \times n)^2$], where CV_{ad} = the CV of the amplification and detection steps. By summing the variances and rearranging, the total assay CV may be written as:

$$CV_{assay} = (1/n)[n(1 + Pq) + (CV_{ad} \times n)^2]^{1/2}$$

The following conclusions can be drawn from this formula. The contribution to the total assay CV from the different steps in the assay depends on the target level. Capture efficiency makes very little difference above five molecules. Below 25 molecules the Poisson contribution dominates the total CV. The accuracy of quantification of target levels between 50 and 500 molecules is significantly affected by the CV of the amplification/detection reaction. With a CV_{ad} of 5%, 100 molecules are detected with a CV of 11–12%; with a CV_{ad} of 30%, the assay CV is 32%.

With 25 molecules in a sample, a 20% CV of quantification is expected in the first pipetting step of the assay. With an amplification/detection CV of 5% the minimum assay CV of quantification would be between 21% (99% efficient capture) and 23% (30% efficient capture). At five molecules, the overall CV will be at least 45%. Thus as the assay detection limit approaches one molecule, it will cease to quantify target precisely well before detectable signals disappear. One solution is to fragment the target in a volume much larger than the assay volume. In this way one target becomes many mini-targets prior to sampling. Target fragmentation should also improve the hybridization efficiency of the probes to the target (due to reduced secondary and tertiary structure) and the capture of the target (due to less steric hindrance).

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A comparison of non-radioisotopic hybridization assay methods using fluorescent, chemiluminescent and enzyme labeled synthetic oligodeoxyribonucleotide probes

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ABSTRACT

N^4-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine-3'-N,N-diisopropyl-methylphosphoramidite has been synthesized. This N^4-alkylamino deoxycytidine derivative has been incorporated into oligonucleotide probes during chemical DNA synthesis. Subsequent to deprotection and purification, fluorescent (fluorescein, Texas Red and rhodamine), chemiluminescent (isoluminol), and enzyme (horseradish peroxidase, alkaline phosphatase) labels have been specifically incorporated. Detection limits of the labels and labeled probes were assessed. Also, the detection limits and non-specific binding of the labeled probes in sandwich hybridization assays were determined. The enzyme modified oligonucleotides were found to be significantly better labeling materials than the fluorescent or chemiluminescent derivatives, providing sensitivities comparable to ^{32}P-labeled probes.

INTRODUCTION

Radiolabeled polynucleotides probes have been extensively employed for the detection of complementary nucleic acids by specific hybridization (1). Within the last few years considerable attention has been given to methods for incorporating non-radioisotopic labels into polynucleotides in order to circumvent the problems inherent to radioactivity. Also, some non-radioactive labeling systems may lead to significantly improved detection limits.

The most important criteria in developing a non-radioactive labeling scheme are maximization of the label density and minimization of non-specific binding (NSB) of the labeled probe. With large polynucleotides (over 100 bases), it is difficult to separate partially or unlabeled material from the fully modified species regardless of the label employed. As a result, typically no purification is attempted after labeling so that the label

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density is dictated only by the yield of the coupling reaction (2,3). However, with short synthetic oligonucleotides the resolution provided by PAGE , HPLC or column chromatography is sufficient to permit separation of fully modified probes from all other reaction components (4-9). The number and position of labeling moieties per polymer can be adjusted to whatever is desired, thus determining the label density.

Small labeled oligonucleotide hybridization probes offer additional advantages over larger polynucleotides. Large quantities (10-100 nanomoles) can be inexpensively produced by chemical synthesis. Since the change in T_m as a function of length is more dramatic with polynucleotides less than 20 bases (10), it is relatively simple to favor the formation of the intended hybrid over mismatches (11).

Most non-radioactive labels are difficult to incorporate directly during the chemical synthesis of oligonucleotides since they are not stable in the reagents employed for synthesis and/or deprotection. As a result, appropriately blocked nucleophilic "handles" such as alkyl- sulfhydryls (12-14) or -amines (4-9) have been incorporated during the solid supported synthesis of DNA. Subsequent to deprotection and purification of the probes, these sites can be used to direct the introduction of nucleophile-specific labeling reagents.

We report here the synthesis of a fully protected 3'-phosphoramidite of an alkylamine derivative of deoxycytidine and its use in the synthesis of oligodeoxyribonucleotides. These alkylamine-containing oligonucleotides have been modified with fluorescent, chemiluminescent and enzyme moieties. Biotinylated oligomers have also been produced and shown to be useful as capture probes in sandwich type nucleic acid analysis. Application of the labeled probes in an assay for a simple DNA analyte suggests that horseradish peroxidase (HRP) and alkaline phosphatase (AP) are superior to the fluorescent and chemiluminescent reporter groups tested.

MATERIALS AND METHODS

Synthesis of the N^4-alkylamino deoxycytidine 3'-phosphoramidite

4-Triazolopyrimidinone nucleoside was synthesized from 5'-O-dimethoxytrityl thymidine as described by Reese et al. (15),

using transient 3'-O-trimethylsilyl protection (16). TLC was performed on silica gel 60 F254 plates in 10% methanol/CH_2Cl_2. Twenty five grams (46 mmoles) of 5'-O-dimethoxytrityl thymidine (R_f = 0.63) was dissolved in 150 ml of dry CH_3CN in a 1 L round bottom flask. After the addition of 50 ml of N,N-dimethylamino-trimethylsilane (Petrarch Systems), the solution was stirred at room temperature for 30 min. The mixture was then evaporated to dryness on a rotary evaporator to give 5'-O-dimethoxytrityl-3'-O-trimethylsilyl-thymidine (compound 1) in quantitative yield (R_f=0.70). 1,2,4-Triazole (51.2 g) was dissolved in 300 ml of CH_3CN and 16 ml of $POCl_3$ was added. The solution was set on ice and upon the dropwise addition of 120 ml of triethylamine, the mixture became a thick slurry. The material was diluted by the addition of 100 ml of CH_3CN. The oily residue of compound 1 was dissolved in 100 ml of CH_3CN and added dropwise to the reaction flask. Stirring was continued for 60 min on ice, then 30 min at room temperature. The solution was diluted with 800 ml of ethyl acetate and extracted twice with each 800 ml of 5% aqueous $NaHCO_3$ and 800 ml of 80% saturated aqueous NaCl. After drying the organic phase over Na_2SO_4, the solvent was removed by evaporation under vacuum at room temperature. Upon co-evaporation with toluene then CH_3CN, 31 g of 4-(1,2,4-triazolo)-1-(β-D-5-O-dimethoxytrityl-3-O-trimethylsilyl-2-deoxyribofuranosyl)-5-methyl-2(1H)-pyrimidinone (compound 2) was obtained and used without further purification (R_f = 0.65; fluorescent blue spot under UV examination).

The 1,2,4-triazole moiety was displaced with ethylene diamine as reported by Sung (17) and Maggio et al. (18). Compound 2 was dissolved in 200 ml of CH_3CN and added to a solution containing 25 ml of ethylene diamine in 200 ml of CH_3CN on ice. After 15 min, the reaction mixture was extracted and dried as above to yield 26.8 g (40.6 mmoles) of 5'-O-dimethoxytrityl-3'-O-trimethylsilyl-N^4-(2-aminoethyl)-5-methyl-2'-deoxycytidine (compound 3) (ninhydrin positive spot at baseline by TLC).

Compound 3, in 200 ml of CH_2Cl_2, was reacted with 100 ml of a 0.5 M solution of N-hydroxysuccinimidyl N-trifluoroacetyl-6-aminocaproate, prepared essentially as described elsewhere (19), at room temperature for 30 min. Subsequent to evaporation of the material to dryness and co-evaporation with 250 ml of toluene,

the resulting foam was dissolved in 400 ml of anhydrous methanol. In order to remove the 3'-O-silyl protection, 55 ml of 1 M K_2CO_3 was added and the reaction was stirred for 15 min at room temperature. At this time, the solution volume was reduced to approximately 500 ml by rotary evaporation. After the addition of 700 ml of ethyl acetate, the mixture was extracted and dried as above to give N^4-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine (compound 4). One half of the crude sample was loaded onto a 1000 ml silica gel 60H column in 100 ml of 0.5% triethylamine in CH_2Cl_2. The column was eluted with 1200 ml, 1200 ml and 1800 ml each of 2%, 4% and 6% methanol in 0.5% triethylamine, CH_2Cl_2, respectively. The 150 ml fractions were monitored by TLC (R_f = 0.37, ninhydrin negative; prior exposure of the plate to ammonia vapor results in a positive ninhydrin test). Fractions containing compound 4 were combined and the nucleoside was precipitated from cold hexanes to yield (from two columns) 21 g (26.4 mmoles; 57% overall yield). ^{1}H-NMR ($CDCl_3$) δ 0.95 (t,2H), 1.3 (m,4H), 1.45 (s,C-5 CH_3), 1.5-1.6 (m,6H), 3.2 (m,2H). UV spectrum (ethanol), lambda max_1 = 246 nm (Σ_{246}=4900), lambda max_2 = 280 nm (Σ_{280}= 5800).

Compound 4 was converted to the corresponding 3'-phosphoramidite using standard procedures (20) to yield N^4-[N-(6-trifluoroacetylamidocaproyl)-2-aminoethyl]-5'-O-dimethoxytrityl-5-methyl-2'-deoxycytidine-3'-N,N-diisopropylmethyl-phosphoramidite (compound 5; Figure 1). ^{19}F-NMR δ -12.5 (relative to CCl_3F). ^{31}P-NMR δ 145.4 and 146.0 (relative to $(CH_3O)_3PO$). 2-Cyanoethyl phosphorus protection has also been utilized. Although the derivative reported here is a 5-methyl deoxycytidine (synthesized from thymidine), we have also produced a deoxycytidine analog (8; synthesized from deoxyuridine).

Oligonucleotide synthesis

All oligodeoxyribonucleotides were synthesized by a solid supported phosphoramidite chemistry (20) on the "home-made" Gene-O-Matic automated DNA synthesizer as described elsewhere (21). The typical thiophenol (for methyl phosphoramidites) and ammonia deprotections were employed (22). Oligomers were purified by PAGE under denaturing conditions (23). Following desalting on C-18 Sep-Pak cartridges (Waters) (24), the oligonucleotides were

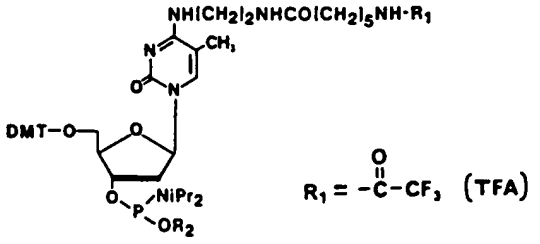

Fig.1. Structure of the fully protected N^4-alkylamino 5-methyl deoxycytidine analog 3'-phosphoramidite. R_2 = methyl or 2-cyanoethyl.

further purified on Sephadex G-25 columns (disposable PD-10 Columns from Pharmacia) equilibrated with water. The concentration of oligonucleotides was determined by UV absorption assuming 1 OD_{260} unit/ 35 µg. All derivatized probe concentrations were determined from the combined label and oligonucleotide absorbance at 260 nm.

All fragments used in the labeling studies were 5'-alkylamine derivatives, 18 or 20 bases long with a G:C/A:T ratio of 1.0 to 1.5. Although not shown here, the methods reported below have also been successfully employed to label oligonucleotides substituted with alkylamino deoxycytidine residues at the 3' end and at internal sites.

Biotin derivatization

Oligonucleotides (3-5 OD_{260} units) were taken up in 100 µl of 0.1 M sodium phosphate, pH 7.5, to which 100 µl of DMF containing 1 mg of "long chain " N-hydroxysuccinimidyl biotin (Pierce Chemicals) was added. After 18 h at room temperature, the mixture was partially purified by Sephadex G-25, as above, and evaporated to dryness. The pellet was diluted with 30 µl of 90% formamide, 0.1% bromophenol blue. The material was loaded onto a 20% polyacrylamide gel (20 x 40 x 0.15 cm) and electrophoresed overnight at 7 ma. Bands were cut out, eluted and desalted as described (23,24).

Capture probe beads

Biotinylated probe (1 nanomole in 66.7 µl of water) was combined with 5 ml of a 0.25% solution (w/v) of 0.8 µm avidin beads (Pandex Laboratories, Mundelein, IL), 1 ml of 20x SSC, 0.5 ml of 1% NP-40 and 0.6 ml of 1 mg/ml poly-A. After 1 h at 37°C,

the beads were washed twice by centrifugation with 4x SSC, 0.1% NP-40 then stored in 2.5 ml of this solution until used.

Microtiter dish wells

Alkylamino probes were covalently bound to passively adsorbed proteins on microtiter dish wells (Immulon II Removawell strips; Dynatech Laboratories, Inc.) as will be described elsewhere (J.A. Running, et al., manuscript in preparation). Coatings were performed in 20 µl.

Fluorescent probes (fluorescein, Texas Red and rhodamine)

For fluorescein derivatization, 2 OD_{260} units of the appropriate oligonucleotide was dissolved in 100 µl of 0.1 M sodium borate, pH 9, containing 2 mg of fluorescein-5-isothiocyanate (Molecular Probes Inc.) and set overnight in the dark. For Texas Red and rhodamine incorporation, 100 µl of 0.1 M sodium phosphate, pH 7.5, was used to dissolve 2 OD_{260} units of the oligonucleotide and 3 mg of Texas Red (sulforhodamine 101 sulfonyl chloride; Molecular Probes) or 5 mg of 5-(and 6-) carboxytetramethylrhodamine succinimide ester (Molecular Probes) in 100 µl of DMF was added. The solutions were vortexed and the reactions were incubated overnight in the dark.

All reaction mixtures were then passed through a 10 ml Sephadex G-25 column previously equilibrated with 30 ml of 10 mM triethylamine acetate, pH 7.3. The colored void volume of the column was concentrated to 50 µl by multiple extractions with n-butanol and dried for 20 min in a Speed Vac concentrator (Savant Instruments). The fluorescent bands (as determined by a hand held UV lamp) were cut, eluted and desalted with a C-18 Sep-Pak cartridge. The probes were stored at 4°C.

Chemiluminescent probe (isoluminol)

ABEI-H [N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinimide; LKB Inc.] was converted to the N-hydroxysuccinimide ester as follows. To 50 mg (133 µmoles) of ABEI-H, 800 µl of a 0.2 M solution of N-hydroxysuccinimde (160 µmole) and 1.33 ml of a 0.2 M solution of dicyclohexylcarbodiimde (266 µmole) were added and thoroughly mixed. After 18 h at 20°C, the solution was centrifuged and the supernatant was removed and evaporated to dryness. The residue was washed twice with diethyl ether then suspended in 100 µl of DMF. This material, ABEI-HSE [N-(4-

aminobutyl)-N-ethyl isoluminol hemisuccinimide, succinimide ester], was stored at -20°C until used.

The isoluminol probe was prepared with 5 mg of ABEI-HSE in 50 µl of DMF, then purified as described above for the Texas Red and rhodamine derivatizations.

HRP derivatization

To 10 OD_{260} units of the appropriate alkylamino oligonucleotide dried in a 1.5 ml Eppendorf tube, 25 µl of 0.1 M sodium borate, pH 9.3, and 500 µl of distilled DMF containing 20 mg of p-phenylene diisothiocyanate (DITC; Pierce Chemicals or Aldrich) were added. The solution was vortexed and incubated in the dark at room temperature for 2 h. After transferring the mixture to a 10 ml centrifuge tube, 3 ml of n-butanol was added. The reaction was vortexed, 3 ml of water was added and the tube was vortexed again. The mixture was centrifuged and the yellowish upper layer was discarded. The extraction process was repeated with further n-butanol additions until a final volume of approximately 50 µl was obtained. Remaining butanol was removed by evacuation, then 10 mg of HRP (Boehringer Mannheim) in 200 µl of 0.1 M sodium borate, pH 9.3, was added. The mixture was vortexed and left overnight at room temperature in the dark.

The HRP-DNA conjugate was separated from free enzyme and oligonucleotide on a 7% polyacrylamide gel. The gel was run under standard conditions until the bromophenol blue was about 2/3 down the gel. The orange-brown bands near the middle of the gel were cut out with a razor blade and put into a 10 ml polypropylene Econo-column (Bio-Rad) to which 3 ml of 0.1 M sodium phosphate, pH 7.5, was added. The conjugate was eluted overnight at room temperature in the dark.

The contents were filtered through the frit at the bottom of the column into a Centricon 10 Microconcentrator (Amicon) prewashed twice with distilled water. The HRP-DNA was then concentrated by centrifugation at 3500 RPM and washed twice with 1 ml portions of 1x PBS. The final product was stored at -20°C.

AP derivatization

Calf intestinal AP (3 mg in buffer; immunoassay grade, Boehringer-Mannheim) was placed in a Centricon 30 Microconcentrator. Approximately 2 ml of 0.1 M sodium borate, pH

9.5, was then added and the device was spun at 3500 RPM until a final volume of 40 µl was obtained. The alkylamino oligonucleotide to be derivatized was then activated with DITC, extracted with butanol and combined with the protein as described above. PAGE, elution (with 0.1 M Tris, pH 7.5, 0.1 M NaCl, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$), and concentration as described for the HRP conjugates were employed. The final product was stored at 4°C.

Preparation of ^{32}P-labeled probes

Fragments (50 pmoles in H_2O) to be labeled were dried by evacuation in 1.5 ml Eppendorf tubes. The probes were resuspended in 15 µl of labeling solution containing 50 mM Tris, pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, 2 units of T_4 polynucleotide kinase (Pharmacia), 100 pmoles of "crude" τ-^{32}P ATP (New England Nuclear), then incubated at 37°C for 90 min. To precipitate the probe, 35 µl of TE (10 mM Tris, pH 7.5, 1 mM EDTA), 2 µl of 1 mg/ml poly-A, 25 µl of 6 M ammonium acetate and 200 µl of ethanol were added. After 1 h at room temperature, the samples were spun at 4°C for 10 min in an Eppendorf centrifuge. The pellets were washed with 80% ethanol and redissolved in 100 µl of TE. The probes were reprecipitated with 10 µl of 3 M sodium acetate and 250 µl of ethanol at -80°C for 30 min, collected and washed as above, then resuspended in 100 µl of H_2O containing 5 µg of poly-A. PAGE analysis revealed better than 99% removal of unincorporated label and over 90% recovery of the probes. Cerenkov counting (1 min) was used for all ^{32}P detections using an LKB Model 1209 Rackbeta Scintillation Counter. Typically, the specific activity ranged between 1000 and 4000 cpm/femtomole of probe.

Sandwich hybridization assay procedure

Assays were conducted either with 10 µl of beads (10 pmoles of bound capture probe) in 1.5 ml Eppendorf tubes or in a microtiter dish well (2 pmoles of bound capture probe) as depicted in Figure 2. Beads were washed by centrifugation and decantation, whereas wells were washed by aspiration.

A stock solution of the target fragment [40 bases; 10 attomoles to 10 pmoles per 20 µl of Hyb Mix (0.1 % SDS, 4xSSC, 1 mg/ml sonicated salmon sperm DNA and poly-A, 10 mg/ml BSA)] was prepared just prior to hybridization. Triplicate samples at each

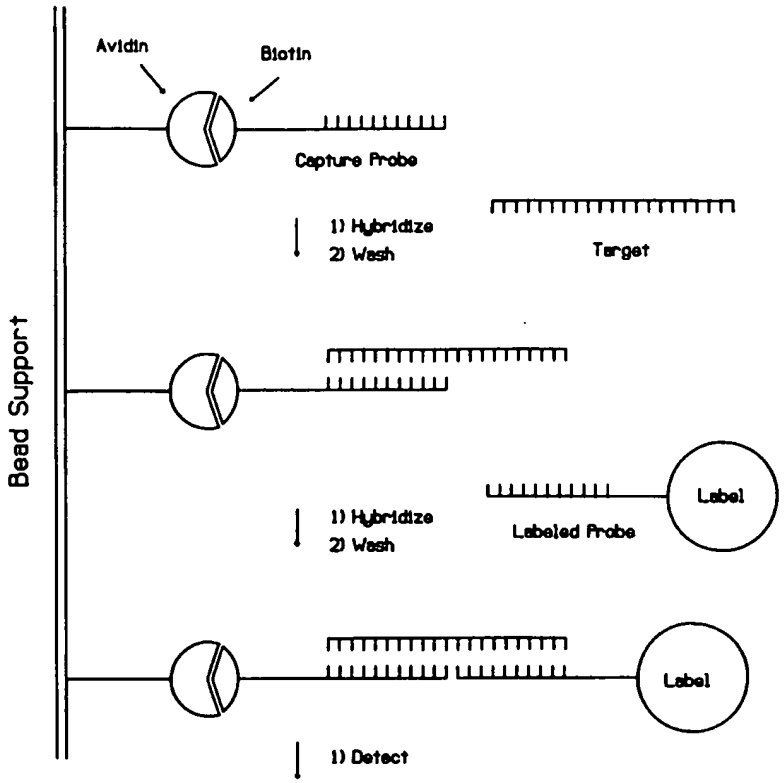

Fig. 2. Two-stage sandwich nucleic acid hybridization assay scheme.

target concentration were employed. After adding 20 µl of target fragment solution to the beads or wells, hybridization was carried out at 55°C in a water bath for 1 h. Tubes were capped and wells were sealed with an adhesive Linbro/Titertek membrane. After washing three times at room temperature with either 0.1% SDS (or 0.1% NP40), 4x SSC for beads or 0.1% SDS, 0.1x SSC for wells, the labeled probe in 20 µl of Hyb Mix was added in a 5-fold molar excess over the maximum target fragment concentration. The solid phase was washed three times as above, then subjected to the required detection solution as described below. A "no target" control (Hyb Mix only) was run in triplicate with each series.

Fluorescent detection

For solution emission spectra of the fluorescent dyes and probes, a Perkin-Elmer MPF-66 spectrofluorimeter was employed.

Excitation and emission wavelengths of 495 nm and 519 nm, 586 nm and 603 nm, 550 nm and 595 nm were used for fluorescein, Texas Red and rhodamine and their derivatives, respectively. These optimal excitation and emission wavelengths were determined from corrected spectra using the Perkin-Elmer survey scan software. All spectra were acquired in 1 cm cuvettes with slits width of 5 nm.

Fluorescent sandwich hybridization assay data was obtained on a Pandex Screen Machine (Pandex Inc., Mundelein, IL) by a modification of the particle concentration fluorescence immunoassay procedure (25). After conducting the hybridizations as described above, the beads were transferred to a 0.2 μm cellulose acetate Pandex microfiltration plate and washed four times with 4x SSC, 0.1% NP-40, then dried thoroughly by vacuum. Fluorescein, rhodamine and Texas Red labels were read according to the manufacturer's suggested settings.

Detection limits for the spectrofluorimeter and Screen Machine were correlated by binding a known quantity of the appropriate dye to 0.8 μm alkylamino polystyrene beads (Pandex). Typically, the Screen Machine was ten times more sensitive.

Chemiluminescent detection of isoluminol

Luminescence of ABEI-H, the isoluminol modified probe, and the dried beads or wells was determined with a hematin catalyst (26) as follows. To the sample, 130 μl of 50 mM NaOH was added and each sample was mixed thoroughly. Subsequently, 20 μl of 0.5 μM hematin (Sigma Chemicals) in 50 mM NaOH was aliquoted into the tubes or wells. After 10 min at room temperature in the dark, 50 μl of 90 mM H_2O_2 was added to each sample just prior to reading on a Turner TD-20e luminometer (Turner Instruments, Mountain View, CA; integration, 15 sec; smoothing, 3). Output was given as the full integral of the light produced during the reaction.

Colorimetric detection of HRP

To each vessel, a 100 μl aliquot of a fresh o-phenylenediamine solution (OPD; in tablet form from Sigma Chemicals; 50 mg dissolved in 5 ml of 50 mM sodium citrate, pH 5.1, containing 3 μl of 30% H_2O_2) was added. After 20 min at 37°C, 50 μl of 4 N H_2SO_4 was added to quench the reaction. For bead assays, the beads were then pelleted by centrifugation and

the supernatant was transferred to a microtiter dish well. The dish was read on a Biotek EL310 plate reader set at 490 nm. Longer incubations did not improve the signal (S) to noise (N) ratios (S/N).

Chemiluminescent detection of HRP

A modification of the enhanced chemiluminescence (ENH/LUM) method (27,28; luminol with p-hydroxycinnamic acid) was employed (9). Luminol (LUM) alone was also employed as a substrate using the same solution without enhancer (no p-hydroxycinnamic acid). For both methods, beads were taken up in 15 µl of chemiluminescent substrate solution, then transferred to 8x50 mm Evergreen polypropylene tubes containing 5 µl of H_2O_2. Microtiter dish wells were treated similarly. After 30 sec, tubes were read on the Turner TD-20e luminometer (delay, 10 sec; integration, 20 sec; smoothing, 20).

Colorimetric detection of AP

A p-nitrophenyl phosphate (NPP) based detection kit (Sigma Diagnostics) was used according to the manufacturer's instructions (1.5 M 2-amino-2-methyl-1-propanol, pH 10.5). Incubations were conducted at room temperature for 1 h and samples were read at 405 nm.

The Blue Gene detection kit (BRL), which employs the nitro blue tetrazolium, 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) system, was also tested. Incubations were carried out until the blue precipitate was visible to the naked eye (4-18 h).

RESULTS

Although we have investigated numerous techniques for the purification of non-radioisotopically labeled oligonucleotides, we have found PAGE to be the most convenient and reliable method. Figure 3 shows the electrophoretic mobility profile of several different labeled oligonucleotides. In each case, all reaction components were well separated. Since the fluorescent and chemiluminescent labeling reagents caused significant sample smearing during PAGE, the reaction mixtures were first passed through a molecular sieving column. Typically, nanomole quantities of probe were modified and purified. Since it is usual that femtomoles of labeled probe are required for hybridization

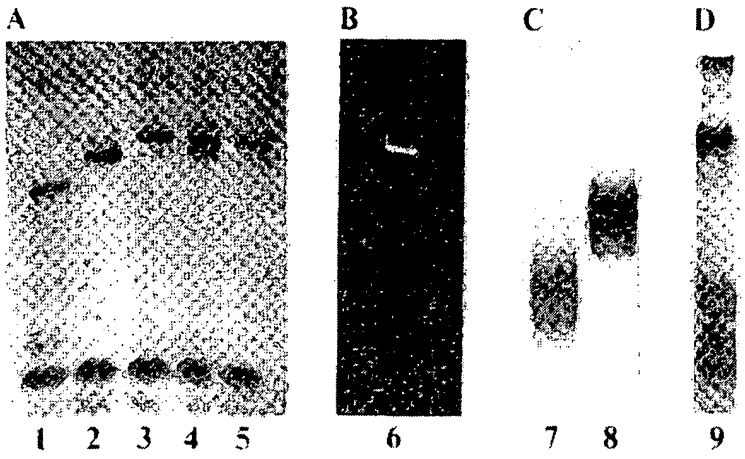

Fig. 3. PAGE analysis of labeled oligonucleotide hybridization probes. Panel A, UV shadowed 20% denaturing gel; panel B, long wavelength UV illumination of the panel A gel; panels C and D, UV shadowed 7% gels. 1, crude 20-base probe; 2, alkylamino derivative of the probe from 1; 3, biotin derivative; 4, isoluminol derivative; 5 and 6, fluorescein derivative; 7, AP probe; 8, AP. In 9, a crude reaction of HRP probe is shown. HRP is in the well, HRP-DNA is at the center of the gel, while unmodified DNA and dye are at the bottom. All fragments are 20 bases in length. In panel A, the lowest band in each lane is bromophenol blue.

analysis (enzyme labels), one synthesis can yield sufficient material for hundreds of thousands of assays.

To determine the detection limits of the various labeled probes in nucleic acid assays, we used a simple two-step sandwich method (29,30) as illustrated in Figure 2. The hybridizations were conducted in an identical manner for each probe using polystyrene beads or microtiter dish wells containing the capture probe. HRP, isoluminol and ^{32}P based assays were conducted on both solid phases. No significant difference was noted in the sensitivity (data not shown). Microtiter dish wells were preferred since the washing steps were considerably simpler to perform.

The assay detection limits (defined as the minimum quantity of analyte that gave a signal to noise ratio, S/N, of 2) are given in Table I. Also given are the detection limits for the labels and labeled probes. In Table II, representative results for sandwich assays performed in triplicate are presented.

Table I
Detection limit determinations.

Detection Limits- (in femtomoles)	Label Alone	Labeled Probe	Probe in Assay
Label (detection system)			
In Sandwich Assay:			
Fluorescein	20	100	500
Texas Red	20	30	100
Rhodamine	5	20	100
Isoluminol	1	30	100
^{32}P	0.05	0.05	0.05
AP (NPP)	5	5	5
AP (NBT/BCIP)	0.2	0.2	0.5
HRP (OPD)	0.1	0.1	0.1
HRP (LUM)	1	1	1
HRP (ENH/LUM)	0.05	0.05	0.05
In HBV assay:			
Fluorescein	-	-	100
HRP (ENH/LUM)	-	-	0.02

See Materials and Methods for abbreviations, assay and detection procedures.

Analyses were performed with and without the target fragment (absolute signal, S, and noise, N, respectively). The "no label" column shows the contribution of the reagents and/or instruments to backgrounds. The difference between N and the "no label" controls is an indication of the amount of NSB of the labeled probe.

Several observations can be made from Tables I and II. For fluorescent (fluorescein and rhodamine) and chemiluminescent (isoluminol) probes, a considerable loss in sensitivity is observed when comparing the labeled probes to the labels alone (Table I). Another significant decrease in sensitivity is realized in the hybridization assays (Table I). This is due not only to quenching upon hybridization, but also to NSB of the

Table II
Sandwich assay results.

	S	N	S/N	no label	[target][5]
Label (detection system)					
Fluorescein[1]	5822 ±493	2325 ±231	2.5 ±0.3	100 ± 50	500
Texas Red[1]	1649 ±183	782 ±138	2.1 ±0.4	100 ± 50	100
Rhodamine[1]	4422 ±347	2221 ±570	2.0 ±0.5	100 ± 50	100
Isoluminol[2]	2.7 ± 0.4	1.2 ± 0.6	2.3 ±1.2	0.10 ±.01	100
AP (NPP)[3]	0.60 ±.02	0.29 ±.01	2.1 ±0.1	0.26 ±.01	10
^{32}P [4]	118 ± 18	36 ± 7	3.2 ±0.8	25 ± 6	0.05
HRP (OPD)[3]	.014 ±.007	.003 ±.001	5 ± 3	.002 ±.001	0.10
HRP(ENH/LUM)[2]	192 ± 7	32.7 ±7.3	5.9 ±1.3	12.7 ±0.5	0.10

See Materials and Methods for abbreviations.1, in absolute fluorescent counts; 2, integral light output in relative luminescent counts; 3, absolute visible absorbance at maximum; 4, Cerenkov counts; 5, in femtomoles.

labeled probes (compare N and "no label", Table II). The NSB apparently arises from the hydrophobic nature of the labels, since, in each case, the fluorescent and chemiluminescent probes gave considerably higher backgrounds than a ^{32}P labeled probe employed at comparable concentrations. On a mole basis, fluorescent and chemiluminescent probes gave 4100-19,100 fold higher NSB than a probe of the same length and composition labeled with ^{32}P (Table II; NSB ≈ [target]x[(N-no label)÷S]; $NSB_{fluorescein}$ = 191 fm; $NSB_{Texas\ Red,\ rhodamine,\ isoluminol}$ = 41-48 fm; $NSB^{32}P$ = 0.01 fm).

On the other hand, binding either HRP or AP to a probe and subsequent hybridization had little effect on the activity of the labels, independent of the detection method. Also, NSB was less problematic. It is noteworthy that AP with NBT/BCIP and HRP with either OPD or ENH/LUM compare favorably in terms of detection limits with ^{32}P as a label. HRP was found to be a more sensitive label than AP by 10 fold (ENH/LUM versus NBT/BCIP, respectively). In addition, the time required for detection is considerably less for HRP (30 sec for ENH/LUM with HRP versus 18 h for NBT/BCIP with AP). For HRP analysis, the relative precision of the luminescent methods was far greater than that of the colorimetric detection system (Table II).

The addition of carrier substances (BSA, sonicated salmon sperm DNA, poly-A) and detergents (NP-40 and SDS) was found to significantly decrease NSB for all the labeled probes employed. Over coating of the polystyrene beads and plates with the same substances has also proved helpful. However, we were unable to fully eliminate analyte-independent binding. Probe length and composition are important criteria in decreasing label-independent NSB. Fragments of 18-20 bases with a G:C content of no more than 60% containing one label were found to be optimal in the sandwich assay method investigated here.

The simple assay described in Figure 2 was compared to the analysis of hepatitis B viral DNA with a solution phase sandwich method as described elsewhere (8) using fluorescein and HRP labels (Table I). Since six labeled probes were used per hepatitis genome (8), a considerable improvement in detection limit was realized while maintaining the relative efficiency of the enzyme versus fluorescent label.

DISCUSSION

N^4-alkylamino deoxycytidine has been introduced into polynucleotides by nick translation with N^4-(6-aminohexyl) dCTP (31), by bisulfite catalyzed transamination (32) and by the coupling of fully protected N^4-alkylamino cytidine 3'-phosphoramidite during automated synthesis (9). There are significant advantages to the incorporation of the cytidine analog through chemical DNA synthesis. The modified nucleotide can be added in any number and at any position within the probe. Large scale synthesis of a variety of non-isotopically labeled derivatives can be conducted readily from commercially available or easily synthesized amine-specific labeling reagents. The use of small single strand synthetic probes offers the opportunity to purify the fully labeled oligonucleotide from partially and unmodified material by PAGE, providing an excellent resolution of all the reaction mixture components. The resulting probes can therefore be labeled at the maximum possible specific activity. Also, the deoxycytidine derivative can be synthesized on a large scale inexpensively and in good yield (57% overall; 8,33).

We did not anticipate a significant destabilization of

hybrids formed with oligonucleotides containing N^4-alkylamino deoxycytidine since upon alkylation of exocyclic amines, K_{eq} for purine-pyrimidine association is not greatly diminished (34). Recently, the T_m determinations of N^4-alkylamino deoxycytidine substituted versus unmodified polynucleotides have shown that duplex stability is essentially unaffected (B. Warner, unpublished results; 31,35).

It has often been noted in immunofluorescent procedures that "over labeling" can lead to non-specific staining (36). The use of fewer than 1 label in 20 nucleotides could perhaps decrease the NSB observed with fluorescent probes. The observed decrease in absolute fluorescence for oligonucleotide derivatives (Table I) is probably similar to that reported for fluorescent labeled antibodies where the presence of aromatic amino acids is known to cause quenching (37). The length and composition of the linker arm to nucleotides may be important, as has been noted for both biotinylated (2) and fluorescein labeled probes (7); however, the 10 atom spacer employed here is near the reported optimum. In the simple hybridization assay system utilized, photobleaching is an unlikely problem, although we did not investigate the addition of retardant compounds such as propyl gallate and p-phenylenediamine (38,39). Since a 10-fold decrease in NSB or quenching would not lead to an adequate detection limit, we did not pursue modification of the fluorescence systems. New instrumentation may greatly improve the detection limits observed with fluorescent probes (5).

The moiety to which isoluminol derivatives are bound can greatly affect the detection limit. For instance, the detection limit reported for the ABEI adduct of thyroxine is 20-100 times poorer than for ABEI alone (40). A similar decrease in sensitivity was observed for the oligonucleotide probe reported here (Table I; 30 fold decrease). The detection limit for isoluminol reported in Table I is consistent with previous reports (26,40).

In contrast to the fluorescent and chemiluminescent labels tested, little loss in sensitivity is noted upon attachment of enzymes to probes or as the result of hybridization of the labeled probes to the solid supported target fragment. Label to

label comparison (Table I) suggests that enzymes such as HRP and AP are the superior non-radioisotopic reporter groups and provide sensitivities nearly equivalent to ^{32}P. Although the detection limits of HRP with the various detection reagents are consistent with previous reports (27,28), our observed limit of detection for AP (enzyme alone and labeled probe) with NBT/BCIP is considerably poorer than reported elsewhere for AP probes used in dot blot formats (Table I, 500 attomoles; Ref 4, 2 attomoles; Ref 13, 24 attomoles). To some extent, the discrepancy could be due to the assay formats and solid phases employed.

Lysine residues are a particularly useful point of attachment of enzymes to oligonucleotides, given their usual abundance and the ease by which specific conjugation can be achieved. Due to the minimal perturbations on the enzymatic activities noted, it seems likely that the site of oligonucleotide incorporation on the HRP and AP labels is remote with respect to the active sites. Occasionally, amine modification can significantly alter enzyme activity; however, several alternative enzyme derivatization strategies can be employed (41).

The use of alternative fluorescent reporters (42) or chemiluminescent moieties (43) may alleviate the shortcomings of the labels used in our studies. Appropriate molecular design that takes into consideration electron density changes upon attachment, hydrophobicity and hydrophilicity, quenching and orientation may eventually lead to adequate small molecular labels. However, many of the best molecular reporting functions for direct labeling (eg, isoluminol) have also been tailored to serve as enzyme substrates (eg, luminol oxidation by HRP). This coupled with the intrinsic amplification provided by enzymes with high substrate turnovers significantly favors enzyme labeling methods. Using the HRP labeling strategy presented here and an amplification system based on chemically cross-linked oligonucleotides, we have been able to detect as little as 0.1 attomoles (60,000 molecules) of hepatitis B virus in human serum samples (9). Enzyme labeling schemes for oligonucleotides may eventually lead to detection limits of less than 100 molecules (44).

In conclusion, facile introduction of fluorescent, chemiluminescent and enzyme labels into synthetic oligonucleotide hybridization probes has been demonstrated using an N^4-alkylamino deoxycytidine derivative. Although reports of the synthesis and use of fluorescent (5-7), chemiluminescent (45), and enzyme (4,8,9,13) labeled oligonucleotide hybridization probes have appeared elsewhere, to our knowledge no direct comparison of the detection limits achievable with these materials has been reported. We have shown that two enzymes commonly employed in immunoassays, HRP and AP, are superior to fluorescein, Texas Red, rhodamine and isoluminol as non-radioisotopic reporter functions in nucleic acid analysis based on the observed detection limits in a simple sandwich assay system.

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